Monoclonal Antibodies Targeting \( \text{Le}^c\text{Le}^x \)-Related Glycans with Potent Antitumor Activity

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Abstract

Purpose: To produce antitumor monoclonal antibodies (mAbs) targeting glycans as they are aberrantly expressed in tumors and are coaccessory molecules for key survival pathways.

Experimental Design: Two mAbs (FG88.2 and FG88.7) recognizing novel tumor-associated Lewis (Le) glycans were produced by immunizations with plasma membrane lipid extracts of the COLO205 cell line.

Results: Glycan array analysis showed that both mAbs bound \( \text{Le}^c \), \( \text{di-Le}^x \), and \( \text{Le}^x \), as well as \( \text{Le}^c \)-containing glycans. These glycans are expressed on both lipids and proteins. Both mAbs showed strong tumor reactivity, binding to 71% (147 of 208) of colorectal, 81% (155 of 192) of pancreatic, 54% (52 of 96) of gastric, 23% (62 of 274) of non–small cell lung, and 31% (66 of 217) of ovarian tumor tissue in combination with a restricted normal tissue distribution. In colorectal cancer, high FG88 glycoepitope expression was significantly associated with poor survival.

Introduction

Successful cancer immunotherapy is dependent on the generation of monoclonal antibodies (mAbs) with good specificity and potency killing. The complexity of the glycome and altered expression of glycosyl transferases associated with malignant transformation make cancer cell-associated carbohydrates excellent targets (1–4). Glycolipids are particularly attractive due to their dense cell-surface distribution, mobility, and association with membrane microdomains, all of which contribute to their participation in a wide range of cellular signaling and adhesion processes (4–6). Generating antiglycolipid antibodies, however, is a challenging task as they do not provide T-cell help and the mAbs are usually low-affinity IgMs.

Lewis (Le) carbohydrate antigens are formed by the sequential addition of fucose onto oligosaccharide precursor chains on glycoproteins and glycolipids through the concerted action of a set of glycosyltransferases (7). Type I chains (containing \( \text{Gal}[1\rightarrow3]\text{GlcNAc} \)) form \( \text{Le}^c \) and \( \text{Le}^v \), whereas type II chains (containing \( \text{Gal}[1\rightarrow4]\text{GlcNAc} \)) form \( \text{Le}^e \) and \( \text{Le}^x \). \( \text{Le}^c \) and \( \text{Le}^x \) antigens are regarded as blood group antigens, yet many human cancers express \( \text{Le}^c \) or \( \text{Le}^x \) antigens regardless of Lewis blood group status (8–10). In addition, \( \text{Le}^e \) and \( \text{Le}^x \) antigen frequently coexist in human tumor cells (11). Dimeric \( \text{Le}^c \) and \( \text{Le}^x \) have also been identified as tumor-associated antigens in breast and gastrointestinal carcinomas (9, 10, 12).

Only a limited number of mAbs recognizing glycans have been described (13, 14). GNX-8 (human IgG1) bound the extended type I chain epitope \( \text{Le}^c\text{Le}^e \) (15) and FC-215 (murine IgM), an anti-\( \text{Le}^c \) mAb that induced transient antitumor responses, but caused profound neutropenia in phase I trials (16, 17). NCC-ST-421 (ST-421, murine IgG3) recognized gastric cancer-associated dimeric \( \text{Le}^x \) and demonstrated significant antitumor effects in a human tumor xenograft model (18). The murine IgM mAb 43-9F, targeted \( \text{Le}^c\text{Le}^x \) epitopes, cross-reacting with simple and extended \( \text{Le}^c \) epitopes, but had no in vivo antitumor reactivity (19). The mAb 504/4 (SC104, murine IgG1), recognized sialyl (S) \( \text{Le}^c \)-related glycans, induced antibody-dependent cellular cytotoxicity (ADCC) and CDC, as well as direct tumor cell death and importantly, demonstrated tumor growth inhibition in vivo (20).

The mAbs demonstrated excellent antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), in addition to direct tumor cell killing via a caspase-independent mechanism. Scanning electron microscopy revealed antibody-induced pore formation. In addition, the mAbs internalized, colocalized with lysosomes, and delivered saporin that killed cells with subnanomolar potency. In vivo, the mAbs demonstrated potent antitumor efficacy in a metastatic colorectal tumor model, leading to significant long-term survival.

Conclusions: The mAbs direct and immune-assisted tumor cell killing, pan-tumor reactivity, and potent in vivo antitumor efficacy indicate their potential as therapeutic agents for the treatment of multiple solid tumors. In addition, internalization of saporin conjugates and associated tumor cell killing suggests their potential as antibody drug carriers. Clin Cancer Res; 21(13): 2963–74. ©2015 AACR.
**Translational Relevance**

Differentially expressed tumor-associated carbohydrate antigens constitute excellent targets for therapeutic antibody development. This report describes the discovery of two monoclonal antibodies (mAbs) that recognize extended non-sialylated Le-containing glycans on tumor glycolipid and glycoproteins with high functional affinity. The FG88 mAbs exhibit comprehensive tumor tissue reactivity combined with limited normal tissue distribution and excellent immune-mediated and direct tumor cell killing via a unique mechanism that may elicit further immune-mediated tumor regression. Furthermore, the mAbs have independent prognostic value in patients with colorectal tumors. These favorable attributes, combined with their potent antitumor activity in a mouse xenograft model suggest promising clinical potential. In addition, the internalization efficiency of the mAbs and their lysosomal colocalization make them attractive candidates for drug conjugation. We anticipate that the excellent preclinical effectiveness of our murine mAbs will translate, following humanization, into therapeutic antitumor efficacy in the clinic.

It is currently in phase I/II clinical trials in gastrointestinal cancer (NCT01447732). More recently, human SLea mAbs were produced using a patient vaccination strategy that showed specific binding to SLea and exhibited ADCC, CDC, and antitumor activity in a xenograft model (21).

In this study, we describe the characterization of two mAbs, FG88.2 and FG88.7, targeting the novel galβ1-3GlcNAcβ1-3Galβ1-4(Fucα1-3)GlcNac (LeβLeα) glycan as well as di-Leα and LeβLeα glyco-epitopes that are expressed by a wide range of tumors but have a restricted normal tissue expression. Both mAbs display effective immune-mediated and direct cytotoxicity that translated into potent in vivo antitumor activity. Furthermore, their efficient internalization suggests potential as antibody drug carriers (ADC).

**Materials and Methods**

**Materials, cells, and antibodies**

Colon rectal (COLO205, HCT-15, and HT29), ovarian (OVCAR3), gastric (AGS), lung (DMS79), T-cell leukemia (Jurkat), and mouse (Balb/c) lymphoblastoid myeloma (NS0) cancer cell lines were all obtained from the ATCC. Colonrectal cell line HCT-15HM2 is a high-metastasizing variant of HCT-15 (22). All cell lines were regularly authenticated using short tandem repeat profiling. mAbs, 7-Le and 225-Le, were from Abcam, anti-CD40 mAb from R&D Systems, anti-FA-44 mAb from Upstate (Millipore), anti-CEACAM mAb from bEbioscience, OKT3 (anti-CD3), FG27.10 (ant-Leβα), FG27.18 (anti-Leβα), 791T/36 (anti-CD55), and 505/4 (SC104; anti-Sdi-Leα) were produced in house. Alpha-galactosylceramide was from Alexis Biochemical, glycan–human serum albumin (HSA) conjugates from IsoSep AB and lipids from Sigma.

**Generation of mAbs**

Plasma membrane lipid extract [0.36 mole%] from 5 × 10⁷ COLO205 cells incorporated into liposomes was used to immunize BALB/c mice at two-weekly intervals over a 2-month period, with α-galactosylceramide and anti-CD40 mAb as adjuvants. Five days after the final immunization, the splenocytes were harvested and fused with N80 myeloma cells. Stable clones were established by repeated limiting dilutions and mAbs purified using standard protein G affinity chromatography.

**Flow cytometry**

Cells (1 × 10⁶) were incubated with primary mAbs at 4°C for 1 hour followed by FITC-conjugated secondary mAb and fixing, as described previously (23) and in Supplementary Materials and Methods. For the propidium iodide (PI) uptake, cells (5 × 10⁶) were incubated with mAbs for 2 hours at 37°C followed by the addition of 1 μg of PI for 30 minutes. Cells were resuspended in PBS and run on a Beckman Coulter FC-500 with WinMDI 2.9 for analysis.

**Thin-layer chromatography analysis of glycolipid binding**

Glycolipid extract from 2 × 10⁶ COLO205 cells (20) spotted onto Merck high-performance thin-layer chromatography (HPTLC) silica plates and developed twice in chloroform: methanol:H₂O (60:30:5) followed by twice hexane:diethyl ether:acetic acid (80:20:1.5). The dried plates were sprayed with 0.1% (w/v) polyisobutylmethacrylate (Sigma) in acetone and blocked with PBS 2% (w/v) BSA (PBS/BSA) for 1 hour at room temperature. The plates were then incubated overnight at 4°C with primary mAbs followed by two 1-hour incubations with biotinylated anti-mouse IgG (Sigma) and IRDye 800CW streptavidin (LIGOR Biosciences), respectively. Plates were air-dried and lipid bands visualized using a LICOR Odyssey scanner.

**SDS-PAGE and Western blot analysis**

Briefly, 1 × 10⁷ or 1 × 10⁶ cell equivalents of cancer cell lysate, total lipid extract, and plasma membrane lipid extract were subjected to SDS-PAGE (4%–12% Bis–Tris NOVEX; Invitrogen), and transferred to Immobilon–FL PVDF membranes (EMDMillipore). Membranes were blocked for 1 hour followed by incubation with primary mAbs. mAb binding was detected using biotinylated anti-mouse IgG (Sigma) and visualized using IRDye 800CW streptavidin (LIGOR Biosciences).

**Lewis antigen and saliva sandwich ELISA**

ELISA plates were coated overnight at 4°C and processed as described in Supplementary Materials and Methods.

**Glycan array analysis**

mAbs were screened for binding to ≥600 natural and synthetic glycans (core H group, version 5.1) by the Consortium for Functional Glycomics (CFG). Slides were incubated with 1 μg/mL of antibody for 1 hour, before detection with Alexa Fluor 488–conjugated secondary mAb.

**Immunohistochemistry assessment**

Normal and tumor tissue binding was analyzed by immunohistochemistry (IHC) as described previously (20). Briefly, after antigen-retrieval, blocking of endogenous peroxidase activity and nonspecific binding sites, the sections were incubated with primary mAbs at room temperature for 1 hour. Primary mAb binding was detected by biotinylated secondary mAb (Vector Labs) followed by preformed
streptavidin-biotin/HRPO (Dako Ltd.) and 3,3'-diaminobenzidine as the substrate. Finally, sections were counterstained with hematoxylin. Staining was analyzed via New Viewer software 2010 and scored with a semiquantitative histologic score (H-score, 0–300) taking into account the staining intensity: negative (0), weak (1), medium (2), and strong (3) and the percentage of positive cells. The stained slides were analyzed by two independent observers and a consensus was agreed. Statistical analysis was performed using SPSS 13.0 (SPSS Inc.). Stratification cutoff points for the survival analysis were determined using X-Tile software (24) and P < 0.05 were considered significant.

Patient cohorts

The study populations include cohorts of consecutive series of 462 archived colorectal cancer (25) specimens (1994–2000; median follow-up, 42 months; censored December 2003; patients with lymph node–positive disease routinely received adjuvant chemotherapy with 5-fluorouracil/folinic acid), 350 ovarian cancer (26) samples (1982–1997; median follow-up, 192 months; censored November 2005; patients with stage II to IV disease received standard adjuvant chemotherapy that in later years was platinum based), 142 gastric cancer (27) samples (2001–2006; median follow-up, 66 months; censored Jan 2009; no chemotherapy), 68 pancreatic and

Figure 1.

A, cell-surface binding of FG88 hybridoma supernatants. Binding was assessed by flow cytometry on COLO205 cells by (i) FG88.2 and (ii) FG88.7 hybridoma supernatants. mAb SC104 (iii) and mouse IgG (iv), both at 3.3 × 10^{-5} mol/L were positive and negative control, respectively. B, FG88 mAbs recognize Le^a,b)-containing glycans on glycolipid and glycoproteins. I, Western blot analysis of FG88.2 mAb (3.3 × 10^{-5} mol/L). Lane 1, COLO205 cell lysates (1 × 10^5 cells); lane 2, COLO205 plasma membrane (1 × 10^5 cells); lane 3, COLO205 total lipid extract (1 × 10^6 cells), and lane 4, COLO205 plasma membrane lipid extract (1 × 10^6 cells). II, HPTLC analysis of FG88.2 binding (3.3 × 10^{-5} mol/L) to cancer cell (2 × 10^6 cells) glycolipid extracts. Lane 1, AGS; lane 2, COLO205. C, ELISA analysis of binding of FG88 mAbs to Lewis glycan–HSA conjugates. The 7-Le^a, 225-Le, FG27.18, and FG27.10 mAbs were included as positive controls for Le^a, Le^b, Le^c, and Le^e, respectively. The negative control consisted of omission of the primary mAb. Error bars represent the mean ± STD of quadruplicate wells (*, P < 0.05; **, P < 0.01; and ***, P < 0.001 versus control, ANOVA followed by the Bonferroni multiple comparisons test, GraphPad Prism 6). D, Glycan array screening of FG88.2 and FG88.7 (CFG, core H, version 5.1). The top five binding glycans are shown as a percentage of the highest binding glycan with glucosylamine (●), galactose (○), fucose (▲), and mannose (◇). Sp denotes the length of spacer between glycan and slide. The complete results for FG88.2 (42) and FG88.7 (43) have been deposited on the CFG website.
120 biliary/ampullary cancer (28) samples (1993–2010: median follow-up, 45 months; censored 2012; 25%–46% of patients received adjuvant chemotherapy with 5-fluorouracil/folinic acid and gemcitabine). 220 non–small cell lung cancers (January 1996–July 2006; median follow-up, 36 months; censored May 2013; none of the patients received chemotherapy prior to surgery but 11 patients received radiotherapy and 9 patients received at least 1 cycle of adjuvant chemotherapy postsurgery) obtained from patients undergoing elective surgical resection of a histologically proven cancer at Nottingham or Derby University Hospitals (Derby, United Kingdom). No cases were excluded unless the relevant clinicopathologic material/data were unavailable.

**Cell viability analysis**

Cells (1 × 10^6/well) were allowed to adhere prior to incubation for 72 hours with increasing concentrations of mAbs in the presence or absence of 20 μM of a pan-caspase inhibitor: carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]fluoromethylketone (Z-FMK-VAD), after which cell viability was analyzed using either ^3^H-thymidine (0.5 μCi/well) during the final 24 hours and its incorporation measured using Microscint 0 liquid scintillant on a TopCount NXT (PerkinElmer) or a WST-8 assay (CCK-8; Sigma-Aldrich). Phase contrast images of mAb-treated HCT-15 cells were obtained with ×10 magnification using a Nikon Eclipse TS100.

**Confocal microscopy**

FG88 mAbs, labeled with Alexa Fluor-488 (495/519 nm) according to the manufacturer’s protocol (Invitrogen), were added to HCT-15 cells (1.5 × 10^5) on coverslips and incubated for 1 hour at 37°C before removing excess mAb with HEPES buffer (Invitrogen). Hoechst 33258 (350/461 nm) nucleic acid stain (1 μg/mL; Invitrogen) and LysoTracker deep red (647/668 nm) lysosomal stain (50 nmol/L; Invitrogen) were added during the last 30 minutes, with Cell Mask Orange (554/567 nm) plasma membrane stain (2.5 μg/mL; Invitrogen) added during the final 10 minutes. Localization of the mAbs was visualized using confocal microscopy through a 63 × 1.4 NA oil objective (ZEISS AX10 Observer Z1; Carl Zeiss) with Zen 2009 image acquisition software.

**ADC assay**

ADC was evaluated by measuring the cytotoxicity of immune-complexed mAbs with a mouse Fab–ZAP secondary conjugate (Advanced Targeting Systems; ref. 29). Cells were plated in triplicates overnight into 96-well plates (2,000 cells, 90 μL/well). After preincubation (30 minutes at room temperature) of a concentration range of FG88 mAbs with 50 ng of the Fab–ZAP conjugate, 10 μL of conjugate or free mAb was added to the wells and incubated for 72 hours. Control wells, consisted of cells incubated without conjugate, incubated with secondary Fab–ZAP without primary mAb and incubated with a control mAb in the presence of Fab–ZAP. Cell viability was measured by ^3^H-thymidine incorporation during the final 24 hours. Results are expressed as a percentage of ^3^H-thymidine incorporation by cells incubated with conjugate compared with primary mAb only.

**ADCC and CDC**

ADCC and CDC were performed as described previously (23). ^5^Cr-labeled target cells (5 × 10^6^) were coincubated with 100 μL of peripheral blood mononuclear cells (PBMC), 10% (v/v) autologous serum or media alone or with mAbs at a range of concentrations (E:T ratio of 100:1). Spontaneous and maximum release (counts per minute (cpm)) was evaluated by incubating the labeled cells with medium or with 10% (v/v) Triton X-100, respectively. The mean percentage lysis was calculated as follows: mean % lysis = (experimental cpm – spontaneous cpm)/maximum cpm – spontaneous cpm) × 100.

**Scanning electron microscopy**

HCT-15 cells (1 × 10^5^) were grown on sterile coverslips for 24 hours prior to mAb (30 μg/mL) addition for 2 or 20 hours at 37°C. Controls included medium alone and 0.5% (v/v) hydrogen peroxide (H2O2; Sigma). Cells were washed with prewarmed 0.1 mol/L sodium cacodylate buffer (SDB) pH 7.4 and fixed with 12.5% (v/v) glutaraldehyde for 24 hours. Fixed cells were washed twice with SDB and postfixed with 1% (v/v) osmium tetroxide (pH 7.4) for 45 minutes. After a final wash with H2O, the cells were dehydrated in increasing concentrations of ethanol and exposed to critical point drying, before sputtering with gold, prior to scanning electron microscopy (SEM) analysis (ISM-840 SEM, JEOL).

**In vivo model**

The study was conducted under a UK Home Office Licence in accordance with National Cancer Research Institute, Laboratory Animal Science Association, and Federation of European Laboratory Animal Science Associations guidelines. Age-matched male MF-1 nude mice (n ≥ 8 for each treatment group; Harlan Laboratories) were implanted intraperitoneally with HCT-15HM2 DLuX cells and tumor establishment monitored by bioluminescent imaging. Mice (bioluminescent signal ≥ 1 × 10^7 p/s) were dosed intravenously (i.v.) biweekly with mAbs (0.1 mg) or vehicle (PBS, 100 μL) until day 120. Bioluminescent intensity (BLI) was evaluated weekly. Briefly, 60 mg/kg o-luciferin substrate was administered to anesthetized mice subcutaneously (s.c.), and BLI...
FG88 mAbs exhibit strong differential tumor/normal tissue reactivity, with colorectal staining being an independent prognostic marker, associated with reduced patient survival.

We assessed the tumor-binding potential of FG88.2 by IHC screening for binding to pancreatic, colorectal, gastric, non-small cell lung (NSCLC), and ovarian TMAs. FG88.2 mAb stained 81% (155 of 192) of pancreatic, colorectal, 71% (147 of 208) of colorectal, 54% (52 of 96) of gastric, 23% (62 of 274) of NSCLC, and 31% (66 of 217) of ovarian tumor tissues and examples of FG88.2 staining level of tumor tissues are shown (Fig. 2A). No significant association between FG88.2 glyco-epitope expression and clinicopathologic variables was observed in any of the tumor TMAs. In the colorectal cancer cohort (25), however, FG88.2 glyco-epitope expression was significantly associated with Bcl-2 ($x^2 = 6.607; P = 0.013$) and with fucosyl transferase 3 (FUT3) expression ($x^2 = 26.712; P = 0.031$). The Kaplan–Meier analysis of disease-free survival of colorectal cancer patients revealed a significantly lower mean survival time in the high FG88.2 glyco-epitope expressing group [mean survival, 36 months (high; $n = 27$) versus 79 months (low; $n = 184$); $P = 0.01$, log-rank test; Fig. 2B]. On multivariate analysis using Cox regression, high FG88.2 glyco-epitope expression in colorectal cancer was a marker of poor prognosis that was independent of stage and vascular invasion ($P < 0.001$).

The strong tumor reactivity of FG88.2 was compared with its normal human tissue distribution (Fig. 2C and Supplementary Table S2). FG88.2 did not bind most normal tissues, including lung, liver (parenchyma), kidney, brain, and cervical, uterine, breast, and salivary glands. Normal tissue staining included columnar epithelium of gall bladder (weak to moderate), bile duct (moderate), thymus (weak), glandular epithelium of colon (moderate), squamous epithelium of tonsil (moderate), and pancreas (weak). Normal human tissue binding by FG88.7 was similar to FG88.2, with the exception of rectum (Supplementary Table S2). On a normal cynomolgous monkey (CN) TMA, FG88.2 exhibited weak staining of skin, colon, ovary, liver, and thymus combined with stronger staining of glandular epithelium of stomach and small intestine (Supplementary Fig. S2A).

As previous studies had shown that Le$^a$ was expressed on neutrophils, the binding of the FG88 mAbs to granulocytes and polymorphonuclear cells (PMNs) from normal donors was analyzed by FACS, where no binding was observed (Supplementary Fig. S2B). In addition, Le$^a$ and Le$^b$ antigens found in tissue secretions can adsorb to erythrocytes. We determined secretory status of 9 healthy human donors by saliva sandwich ELISA (Supplementary Fig. S2C), followed by binding analysis of the FG88 mAbs to erythrocytes from a Le$a^+$-positive donor. Neither FG88 mAb bound to erythrocytes (Supplementary Fig. S2D).

As it is difficult to use primary tumors for cytotoxic experiments due to their high rate of spontaneous apoptosis, we screened cancer cell lines as models for FG88 mAb binding and killing. High FG88.2-binding cancer cells comprised COLO205 and HCT-15 [geometric mean (Gm) $\geq 1,000$ at saturating mAb concentration]. The antigen density (SABC) was calculated to be 618,000 and 902,000 for HCT-15 and COLO205, respectively. Moderately binding cells (Gm $\geq 100$) included HT29 and OVCAR3 (SABC: 88,000 and 23,000, respectively) and low-binding cells (Gm $< 100$) encompassed AGS and DMS79 (SABC: 12,000 and 10,000, respectively; Fig. 2D).
FG88 mAbs mediate direct tumor cytotoxicity via a unique caspase-independent mechanism

In order to ascertain whether cancer cell binding by the FG88 mAbs affected cell viability, we analyzed mAb-induced PI uptake that reflects compromised membrane integrity and ensuing loss of cell viability (Fig. 3A and Supplementary Fig. S3A). FG88.2 induced dose-dependent PI uptake, with strong binding cells, such as HCT-15 and COLO205, being more susceptible than the moderate to weak FG88-binding cells. In addition, proliferation analysis (WST8-based) of mAb-treated HCT-15 cells revealed a dose-dependent growth inhibition with an IC_{50} of 1.04 × 10^{-8} mol/L (Supplementary Fig. S3B).

Classical extrinsic apoptotic cell death involves activation of effector caspases. We thus evaluated caspase activity in FG88.2-induced cytotoxicity via cellular proliferation analysis in the presence and absence of the cell permeant pan-caspase inhibitor Z-FMK-VAD. FG88.2 binding to HCT-15 cells resulted in a dose-dependent decrease in cellular proliferation that was not affected by the presence of Z-FMK-VAD, suggesting no involvement of caspase activity (Fig. 3B). In contrast, Z-FMK-VAD prevented Fas mAb-mediated apoptosis of Jurkat cells under similar conditions (data not shown). Another hallmark of apoptotic cell death is endonuclease-induced DNA fragmentation. FG88.2 did not induce DNA fragmentation in HCT-15 cells, in contrast to the

Antitumor Activity of Novel Glycan Monoclonal Antibodies

Figure 3.
Direct cytotoxic activity of FG88 mAbs. A, dose-dependent increase in PI uptake by a range of tumor cell lines as a result of FG88.2 binding (7.4 × 10^{-8}-2 × 10^{-7} mol/L). B, dose-dependent growth inhibition ([H]-thymidine incorporation) of HCT-15 cells by FG88.2 (2.2 × 10^{-8}-2.7 × 10^{-9} mol/L) in the presence or absence of Z-FMK-VAD (20 μmol/L). Nonlinear regression was performed using GraphPad Prism 6. C, phase-contrast imaging of FG88 mAb-treated HCT-15 cells. Images (magnification, ×10) showing HCT-15 cells after incubation with FG88.2 and FG88.7, both at 1.3 × 10^{-7} mol/L, and RPMI medium (negative control). D, SEM analysis of FG88.2-induced ultrastructural cellular changes. HCT-15 cells were treated with FG88.2 mAb (2 × 10^{-7} mol/L) for 2 or 20 hours. Medium alone (RPMI) and 0.5% (v/v) H₂O₂ were used as negative and positive controls, respectively. Arrows indicate mAb-induced pores.
anti-Fas mAb that resulted in Z-FMK-VAD–sensitive DNA fragmentation in Jurkat cells (Supplementary Fig. S3C). The absence of caspase involvement combined with a lack of DNA fragmentation suggests that FG88.2 induces a direct cytotoxic effect via a distinct nonapoptotic mechanism.

Next, light microscopy and SEM were used to evaluate FG88.2-induced ultrastructural changes. Light microscopy evaluation of mAb-treated HCT-15 cells showed evidence of monolayer disruption, cell rounding, and clumping within 24 hours of mAb addition and a decrease in cell numbers between 24 and 48 hours after mAb addition. This was maintained over the 72-hour incubation period (Fig. 3C). FG88.2-treated HCT-15 cellular aggregates displayed a loss of surface microvilli and the formation of membrane blebs and surface wrinkles (Fig. 3D). Importantly, cell-surface pore formation suggests a cell death mechanism reminiscent of oncosis. Heterogeneous pore sizes with diameters ranging from 0.2 to 1 μm were observed after 2 hours as well as 20 hours. FG88.7 displayed similar characteristics to FG88.2 with respect to cell binding and cytotoxicity (data not shown).

FG88 mAbs internalize efficiently

Confocal microscopy of Alexa Fluor 488–labeled FG88.2 binding to HCT-15 cells over a 2-hour period showed efficient internalization of a proportion of the mAbs (Fig. 4A). In addition, the FG88 membrane staining pattern suggests heterogeneous distribution of the glyco-epitope in the HCT-15 plasma membrane. Over time, internalized FG88 mAbs colocalized with lysosomal compartments (Fig. 4A). Similar results were obtained with FG88.7 (data not shown). Importantly, internalization was validated through toxicity of Fab–ZAP-FG88 immune complexes containing saporin (29). Internalization of the Fab–ZAP–FG88.2 and Fab–ZAP–FG88.7 complexes, but not the Fab–ZAP alone or the Fab–ZAP preincubated with a control mAb (data not shown), led to a dose-dependent (IC50 10^{-14} mol/L) decrease in cell viability of the high glyco-epitope–expressing HCT-15 cells (Fig. 4B). The moderately binding HT29 cells were more refractory.

FG88 mAbs exhibit excellent immune-mediated cancer cell killing (ADCC and CDC) in vitro

The ability of the FG88 mAbs to induce COLO205 tumor cell death in the presence of human PBMCs through ADCC was investigated. Both FG88 mAbs induced potent cell lysis of the high-binding COLO205 cells in a concentration-dependent manner with EC50 values of 10^{-9} mol/L and near 100% lysis at 7.4 × 10^{-9} mol/L (Fig. 5A). Next, we analyzed a range of tumor cell lines for their susceptibility to FG88-mediated ADCC. The FG88 mAbs significantly lysed the high glyco-epitope–expressing COLO205 and HCT-15 above the killing observed with PBMCs alone (Fig. 5B). The mAb 791T/36, a murine IgG2b that cannot bind human CD16 (30), showed no significant killing over the background observed with PBMCs alone, in the majority of cell lines. PBMC killing in the absence of FG88 mAbs was highest for cell lines lacking MHC-I, such as HCT-15 and AGS, and probably reflects NK killing. Noticeably, less immune-mediated killing was seen with the FG88 mAbs on the moderate to weak binding HT29 and DMS79 cells even at high mAb concentration 6.7 × 10^{-8} M. The low-binding OVCAR3 and AGS cells were refractory.

In addition, the FG88 mAbs were very efficient at inducing CDC of high-binding HCT-15 cells in the presence of human PBMCs.
complement, with nanomolar EC50 and over 80% lysis at 7.4 × 10−9 mol/L mAb (Fig. 5C). The FG88 mAbs displayed significant CDC activity against COLO205 cells and to a lesser degree DMS79 cells (Fig. 5D). No or little CDC was seen on the low- to moderate-binding cell lines HT29, OVCAR3, and AGS (data not shown).

In order to relate tumor staining by IHC to the antigen density requirement for cell killing, HCT-15 xenograft tumor tissue was stained with FG88.2 (data not shown). From this analysis, the antigen density required for cell killing by our mAbs was comparable with a score of 2 to 3 (moderate to strong) in the IHC analysis of the tumor tissues and accounts for 39% to 53% of gastrointestinal cancers (Supplementary Table S3). Some of the normal gastrointestinal tissues also showed weak to moderate staining but this is mainly apical cells and it is unclear if mAb will be accessible to these cells.

**Potent in vivo antitumor activity by the FG88 mAbs in a human hepatic metastasis xenograft model**

The mouse HCT-15HM2 DLuX human hepatic metastasis tumor model was used to investigate the antitumor activity of the FG88 mAbs. The HCT-15HM2 DLuX cell line is a bioluminescent variant of a liver metastasizing HCT-15 cell line. Carcinoma establishment, growth, and metastasis were assessed non-invasively via optical imaging. FG88 mAb treatment was initiated in vivo the most aggressive cancers would benefit from FG88.2 therapy. Earlier work has demonstrated the prognostic value of Lex, SLex, and SLea expression in colorectal carcinomas and the association of SLea expression with increased metastatic potential (31–34), but our study is the first to demonstrate independent prognostic

![Image](https://example.com/figure5.png)

**Figure 5.**

*In vitro* immune effector activity of FG88 mAbs. A. dose-dependent ADCC activity of FG88 mAbs on COLO205 cells. 51Cr-labeled COLO205 cells were coincubated with increasing concentrations of FG88 mAbs and human PBMC (E:T of 100:1). B. ADCC activity of FG88 mAbs on a panel of tumor cell lines. FG88 mAbs and control (791T/36) were used at 6.7 × 10−8 mol/L. C. dose-dependent CDC activity of FG88 mAbs on HCT-15 cells. The 51Cr-labeled HCT-15 cells were incubated in the presence of increasing concentrations of FG88 mAbs in the presence of human serum. D. CDC activity of FG88 mAbs on a panel of tumor cell lines. FG88 mAbs and control (791T/36) were used 6.7 × 10−8 mol/L. Significance (B and D) versus PBMC or serum control, respectively, was established by ANOVA followed by the Bonferroni multiple comparisons test, GraphPad Prism 6.
value for Le<sup>A</sup> expression in colorectal cancer as defined by our FG88.2 mAb.

Some of the normal gastrointestinal tissues also showed weak to moderate staining but this is mainly apical cells and it is unclear if mab will be accessible to these cells. Indeed, SC104 mab showed similar staining but showed not toxicity in primate models and no toxicity in clinical trials (NCT01447732, SC104/CEP-37250/KHK2804). Lewis antigens are only synthesized by primates due to antitumor activity of FG88.7 and FG88.2. The HCT-15HM2 DLuX mouse xenograft model was characterized by the growth-inhibitory effect of the FG88 mAbs was characterized by a significant reduction in percentage tumor growth (ventral bioluminescent signal) due to antitumor activity of FG88.7 and FG88.2. The HCT-15HM2 DLuX mouse xenograft model was used to compare the antitumor effect of the FG88 mAbs to vehicle control (group size n > 8). MF-1 nude mice received mAbs (100 μg) i.v. biweekly, treatment was initiated 10 days after tumor inoculation and the final dose was administered on day 120 (arrow).

In contrast, SC104, which recognizes sialylated Le<sup>A</sup>-related glycans, was also cytotoxic to high-binding cells, such as HCT-15 and COLO205, but with a higher IC<sub>50</sub> (2 × 10<sup>−7</sup> mol/L) and via classical apoptosis with evidence of caspase activation (20).

Two independent approaches demonstrated efficient internalization and lysosomal localization of a proportion of the FG88 mAbs in a glyco-epitope density-dependent manner. This indicates their potential clinical usefulness for ADC development. Furthermore, a fraction of the FG88 mAbs remains at the cell surface where their slow dissociation kinetics enables excellent immune-effector functions (ADCC and CDC). Importantly, moderate- to weak-binding cells were less susceptible to ADCC and CDC, offering a further level of protection.

Carbohydrate-recognizing mAbs generally exhibit weak glycan affinity. In contrast, the FG88 mAbs displayed strong functional affinity for a high-density Le<sup>A</sup>-HSA glycoconjugate but nanomolar functional affinity for tumor cell lines overexpressing the Le<sup>A</sup>-containing glyco-epitope such as COLO205 and HCT-15. This probably reflects the more complex binding behavior of the mAbs at the cell surface where the mixed glyco-epitope (glycolipid and glycoprotein) recognition, the fluid membrane, or the potential for internalization and cell killing can explain the higher cell-surface K<sub>d</sub>. Preliminary studies suggest that the glycolipids are responsible for the direct cell killing and ADCC/CDC, requiring high levels of mAb, 10<sup>−5</sup> and 10<sup>−6</sup> mol/L, respectively. In contrast, internalization occurs predominantly via glycoproteins and at subnanomolar (10<sup>−11</sup> mol/L) concentrations of mAb. This suggests that targeting glycans shared between glycoproteins and glycolipids may be ideal for ADC and direct/immune effector cell killing, respectively; potentially debulking the tumor via ADC followed by removing residual disease with mAb alone.

The excellent in vitro cytotoxicity of both mAbs translated in potent antitumor efficacy and significant survival improvement in a colorectal hepatic metastasis xenograft model in which mAb treatment was initiated 10 days after tumor initiation and development of liver metastasis. The FG88 mAbs cured both intra- peritoneal disease and liver metastases in 30% of animals. Postmortem imaging analysis of those animals showed very limited evidence of tumor regrowth. Previous xenografts studies have

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**Figure 6.**

*In vivo* activity of FG88 mAbs in the mouse colorectal hepatic metastasis model. A, reduction in percentage tumor growth (ventral bioluminescent signal) by FG88 mAbs. FG88.2 and FG88.7 significantly reduced tumor growth by day 59 (P = 0.016) and day 65 (P = 0.046), respectively, compared with the vehicle (PBS) control (Mann-Whitney U test). B, survival benefit due to antitumor activity of FG88.7 and FG88.2. The HCT-15HM2 DLuX mouse xenograft model was used to compare the antitumor effect of the FG88 mAbs to vehicle control (group size n > 8). MF-1 nude mice received mAbs (100 μg) i.v. biweekly, treatment was initiated 10 days after tumor inoculation and the final dose was administered on day 120 (arrow).

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shown good antitumor efficacy when treatment was initiated at the time of tumor implantation or shortly afterwards (18, 41). No studies, to date, have shown tumor eradication of 10-day established tumors and liver metastases. SC104 inhibited tumor growth in vivo, but was more effective in the tumor prevention model and only worked in the therapeutic models in combination with 5-FU/leucovorin (21). The humanized SC104 (NCT01447732) currently in phase II trials (SC104/CEP-37250/KHH2804) has been defucosylated for enhanced effector functions. Further studies have been initiated to evaluate the therapeutic potential of human IgG1 chimeric versions of the FG88 mAbs and the initial results are encouraging.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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